REMARKS

The specification is amended to reflect that, as indicated on the Declaration submitted originally with this application when it was filed, this application claims the benefit of the prior-filed provisional application. No new matter is added. It is requested that the amendment be made of record in the file.

Claims 27 and 38-71 were pending in this application. In order to expedite the prosecution of the present application and without conceding to the validity of the Examiner's rejections, Applicants have canceled claims 39-41, 43-45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, and 71, without prejudice to Applicants' right to pursue the subject matter in related applications, and have amended claims 46, 48, 54, 56, and 70 to correct claim dependencies necessitated by the cancellation of claims. Claim 64 has been amended and new claims 72-75 have been added to clarify that which Applicants regard as the invention. Specifically, claim 64 has been amended to recite that the cancers to be treated can include colon cancer, colorectal cancer and melanoma. Support for this amendment can be found in the specification, for example, at page 27, lines 19-37 and page 1, lines 25-37. New claims 72 and 73 are directed to methods for treating cancer comprising administering to a subject in need thereof an effective amount of a nucleic acid molecule comprising a nucleotide sequence encoding IL-12 and an effective amount of 4-1BB ligand, wherein the nucleic acid molecule is introduced into a cell prior to administration of the cell to the subject. New claims 74 and 75 are directed to methods for treating cancer wherein the cell is a fibroblast, monocyte or progenitor cell obtained from bone marrow. Support for the amendments to claims 72-75 can be found in the specification, for example, at page 30, lines 27-30 and page 32, line 9 to page 33, line 14. No new matter has been added by these amendments. Upon entry of this Amendment, claims 27, 38, 42, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, and 72-75 will be pending.

Entry of the foregoing amendments and consideration of these remarks are respectfully requested.

I. THE REJECTIONS UNDER 35 U.S.C. § 112, FIRST PARAGRAPH, SHOULD BE WITHDRAWN

A. The Rejection of Claims 38-71 Under 35 U.S.C. § 112, First Paragraph for Lack of Written Description and Lack of Enablement has been Obviated

Claims 38-71 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of

the claimed invention and as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Specifically, the Examiner contends that the description of wild type IL-12 or 4-1BBL is insufficient to claim all their derivatives because the specification fails to teach the structure-function relationship for the wild-type molecules.

In order to expedite the prosecution of the application and without conceding to the validity of the Examiner's rejection, Applicants have canceled claims 39-41, 43-45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69 and 71, without prejudice to Applicants' right to pursue the subject matter in related applications. Applicants specifically reserve all right to pursue any subject matter removed from the pending claims in a continuation application. The remaining claims do not recite a fragment, analog or derivative and, thus, are believed to have an adequate written description and to be fully enabled.

In view of the foregoing, Applicants respectfully assert that the rejection of claims 38-71 under 35 U.S.C. § 112, first paragraph, for lack of written description support and lack of enablement has been obviated and should be withdrawn.

B. The Rejection of Claims 38-71 Under 35 U.S.C. § 112, First Paragraph for Lack of Enablement has been Obviated

Claims 38-71 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was allegedly not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Specifically, the Examiner contends that the specification fails to provide enablement for routes of administering IL-12 encoding nucleic acid molecules other than intratumoral administration. The Examiner states that the anti-tumor effects described in the specification and the prior art of record are all achieved by local administration. For the reasons detailed below, Applicants respectfully assert that the rejection 35 U.S.C. § 112, first paragraph, for lack of enablement cannot stand and should be withdrawn.

Applicants respectfully assert that the specification of the present application coupled with information known as of the effective filing date of the present application provides sufficient guidance to enable one of skill in the art to administer the nucleic acid compounds of the present invention by any route, without undue experimentation. The specification of the present application provides examples of a variety of routes for administering the nucleic acid compounds recited in the claims (*see*, *e.g.*, the specification, page 35, lines 2-4). Moreover, there are numerous examples in the literature of IL-12 adenoviral vectors administered at sites remote from

a tumor achieving a therapeutic effect. For example, an adenoviral IL-12 vector has been delivered intraperitoneally for the treatment of melanoma lung metastases (Hirschowitz et al., 1999, Am. J. Respir. Cell Mol. Biol. 20:935-941; **Ref. A01**) and intravenously for the treatment of hepatic metastases (Siders et al., 1998, J. Immunol. 160:5465-5474; **Ref. A02**). Thus, contrary to the Examiner's assertion, Applicants respectfully assert that one of skill in the art would be able to deliver the nucleic acid compounds of the present invention using routes of administration at sites remote from the tumor. Accordingly, Applicants respectfully submit that the specification of the present application fully enables one of skill in the art to practice the full scope of the claimed methods.

In view of the foregoing, Applicants respectfully assert that the rejection under 35 U.S.C. § 112, first paragraph, for lack of enablement cannot stand and should be withdrawn.

II. THE REJECTION UNDER 35 U.S.C. § 103 SHOULD BE WITHDRAWN

Claims 38, 39, 42, 46, 48, 50, 52, 54, 55, 58-67 and 70 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Caruso *et al.*, 1996, *Proc. Natl. Acad. Sci. USA* 93:11302-11306 ("Caruso") taken with Melero *et al.*, 1998, *Eur. J. Immunol.* 28:1116-1121 ("Melero") and Vinay *et al.*, *Semin. Immunol.* 10:481-489 ("Vinay"). The Examiner contends that since each of the cited references teaches using more than one agent for cancer therapy, it would have been *prima facie* obvious to combine these agents to generate a new composition for the treatment of cancer with a reasonable expectation of success. Applicants respectfully traverse.

The Examiner asserts that since both the functionality of IL-12 and 4-1BBL are related to the induction of IFN-γ secretion and both IL-12 and 4-1BBL have been demonstrated to have anti-tumor activity, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to combine the teaching of Caruso with the teaching of Melero to use both IL-12 and 4-1BBL for reducing tumor volume with decreased toxicity caused by IL-12 alone and with reasonable expectation of success.

As rebuttal evidence, Applicants respectfully submit that unexpected results are provided within the specification. "Evidence of a greater than expected result may ... be shown by demonstrating an effect which is greater than the sum of each of the effects taken separately (i.e., demonstrating 'synergism'). *Merck & Co. Inc. v. Biocraft Laboratories Inc.*, 874 F.2d 804, 10 USPQ2d 1843 (Fed. Cir.), *cert. denied*, 493 U.S. 975 (1989)." See MPEP § 716.02(a). As demonstrated in the Examples at pages 38-44, co-administration of a recombinant adenoviral vector expressing IL-12 and 4-1BB ligand, as well as co-administration of a recombinant vector expressing IL-12 and a recombinant vector expressing 4-1BB ligand, resulted in a statistically

significant increase in survival in hepatic tumor models (see Figs. 3 and 4). See the specification, at page 42, lines 3-24. Applicants were the first to demonstrate this synergistic effect of the combination which results in an efficacious effect requiring at least 10 fold less IL-12 than the effective dose of IL-12 alone. See the specification at page 44, lines 21-25. These results are far greater than would be expected from the combination of two anti-cancer agents which act by increasing IFN-γ secretion.

Moreover, subsequent to filing this application, Applicants and their colleagues have published results in accord with this invention demonstrating that a combination of IL-12 gene therapy and a co-stimulatory molecule, e.g., an 4-1BB monoclonal antibody, had synergistic effects and reduced the effective dose of IL-12 up to 18-fold, while achieving better efficacy than the individual treatments at the highest dose. Attention is directed to Exhibit A submitted herewith: Chen *et al.*, "Rejection of Disseminated Metastases of Colon Carcinoma by Synergism of IL-12 Gene Therapy and 4-1BB Costimulation", 2000, Mol Ther. 2:39-46 **(Ref. AM** of record). As shown in Exhibit A, synergistic effects of the combination therapy were further demonstrated resulting in a "persistent antitumor response that results in complete remission and long-term survival ...." See Exhibit A at p. 40, col. 1; p. 41, col. 1; p. 41, col. 2; and p. 44, col. 2. Thus, Applicants respectfully submit that even assuming *arguendo* there were *prima facie* case of obviousness, such has been clearly and convincingly overcome.

In view of the foregoing, Applicants respectfully assert that the rejections under 35 U.S.C. § 103(a) cannot stand and should be withdrawn.

CONCLUSION

Applicants respectfully request that the foregoing amendments and remarks be entered and made of record in the present application. Withdrawal of all of the rejections and consideration of the amendments are requested. An allowance of the application is earnestly sought. If any issues remain, the Examiner is respectfully invited to telephone the undersigned.

Respectfully submitted,

Date: May 7, 2004

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doi:10.1006/mthe.2000.0086, available online at http://www.idealibrary.com on IDEAL

ARTICLE

Rejection of Disseminated Metastases of Colon Carcinoma by Synergism of IL-12 Gene Therapy and 4-1BB Costimulation

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Received for publication February 11, 2000, and accepted in revised form May 19, 2000

In an orthotopic model of metastatic colon carcinoma established in the liver of mice, we have previously shown that the natural killer (NK) cells were the major effectors after intratumoral delivery of a recombinant adenovirus expressing the murine IL-12 gene. However, tumor cure and long-term survival were achieved only in a minority of animals. In the present study, we generated an effective antitumoral CD8+ T-cell response by the combination of IL-12 gene therapy and systemic delivery of an agonistic monoclonal antibody against 4-1BB, a costimulatory molecule expressed on activated T cells. In the IL-12 plus anti-4-1BB combination treatment, the effective dose of IL-12 could even be reduced even up to 18-fold and still achieved a better efficacy than the maximal dose of either treatment alone. We further demonstrate that the innate and the adaptive antitumoral immune responses were synergistic, as animals bearing hepatic as well as multiple pulmonary metastases were quantitatively cured of their diseases after IL-12 gene therapy + anti-4-1BB combination treatment. Both NK and CD8+ T cells were necessary in maintaining the long-term antitumor immunity, as depletion of either cell type in the cured animals abolished their abilities to reject tumor cells implanted at distal sites. These results indicate that synergism between innate and adaptive immune responses may be effectively exploited to treat patients with metastatic diseases.

Key Words: **colon metastases; IL-12; 4-1BB; gene therapy; immune modulation.**

INTRODUCTION

Colorectal carcinoma is second only to lung cancer as the cause of cancer deaths. Prognosis for patients with metastatic disease in the liver and other organs is poor. With current treatment, the mean survival time is 37 months (1, 2).

Cancer immunotherapy is a promising approach to combating metastatic diseases by stimulating a systemic antitumor response against disseminated tumor cells in the host. One of the most promising reagents to date is interleukin-12 (IL-12).[2] It plays an important role in orchestrating the host immune response by inducing interferon-γ (IFN-γ) expression, promoting T-helper-1 (Th-1) cell differentiation, and enhancing T-, natural killer- (NK), lymphokine-activated killer cell- (LAK), and macrophage-mediated cytolytic activity (3–9). In our previous study, intratumoral high expression of the adenoviral-mediated IL-12 (Adv.mIL-12) gene induced a strong antitumor immune response in a low immunogenic colon carcinoma (MCA26) liver metastases model in syngeneic BALB/c mice (10). We found that NK cells were the early and major effectors responsible for this Adv.mIL-12 response. However, the early NK response alone was not sufficient in sustaining long-term immunity; in order to achieve long-term survival, a systemic and effective long-term cytolytic T-cell response is also required (11).

4-1BB is a member of the tumor necrosis factor receptor superfamily (12, 13) that binds to a high-affinity 4-

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[2]Abbreviations used: Adv.mIL-12, recombinant adenovirus expressing murine IL-12; APCs, antigen-presenting cells; CTL, cytotoxic T lymphocyte; DL312, E1-deleted control adenovirus; IFN-γ, interferon gamma; LAK, lymphokine-activated killer cells; MNCs, mononuclear cells; NK, natural killer cells; pfu, plaque-forming units.

1BB ligand expressed on antigen-presenting cells (APCs), such as dendritic cells, macrophages, and activated B cells (14, 15). Expression of 4-1BB is restricted to primed CD4+ and CD8+ T cells (16, 17) after antigen or mitogen stimulation and to IL-2-activated NK cells (18). Upon interaction with 4-1BB ligand, 4-1BB provides a strong signal for expansion of the TCR-ligated T cells, especially for the CD8+ T cells *in vivo* (19). It has also been shown that the systemic administration of a 4-1BB agonistic monoclonal antibody results in substantial regression of subcutaneous tumors and that both CD4+ and CD8+ T cells are involved in the antitumor response (20). In this study, we investigated the potential effect of 4-1BB on the IL-12-activated NK-cell, NKT-cell, and T-cell development. We hypothesize that activation of T cells through the 4-1BB costimulatory pathway may further improve the therapeutic effect of IL-12, and the combined effect of IL-12 and 4-1BB may generate a long-lasting antitumor immunity. Interestingly, the results indicate that agonistic anti-4-1BB antibody acts synergistically with Adv.mIL-12 gene therapy to induce a persistent antitumor immune response that results in complete remission and long-term survival of animals bearing liver and lung metastases of colon carcinoma.

MATERIAL AND METHODS

Vector construction (10). A recombinant adenovirus expressing mIL-12 was constructed by replacing the E1A region of the adenovirus type 5 with an expression cassette pAd/RSV-mIL-12 containing the two IL-12 cDNA subunits, p35 and p40, linked by an internal ribosomal entry site (IRES) of the encephalomyocarditis virus. The recombinant virus was generated by cotransfecting pAd/RSV-mIL-12 and pBHG10 into 293 cells. Large-scale production was accomplished in 293 cells and purified by double cesium chloride gradient ultracentrifugation. The viral titer [plaque-forming units (pfu)/ml] was determined by plaque assay in 293 cells. Bioactivity was determined by ELISA, detecting for IFN-γ production by naive splenocytes after coculturing with Adv.mIL-12-transduced MCA26 supernatant.

Liver tumor model and therapeutic protocol. MCA26 is a chemically induced colon carcinoma in BALB/c mouse (21). Metastatic colon cancer was induced by injecting 7×10^4 cells into the left lateral lobe of the livers of 8- to 10-week-old female BALB/c mice (Taconic Farms, Germantown, NY) (10). At day 7, mice with liver tumors measuring 5×5 mm^2 in diameter were selected and given different doses of Adv.mIL-12 or control DL312 (E1A-deleted control adenoviral vector) intratumorally. At days 8 and 10 following tumor implantation, 50 μg of agonistic monoclonal anti-4-1BB antibody (Bristol-Myers Squibb, Princeton, NJ) or control rat Ig (Caltag Laboratories, Burlingame, CA) was given ip.

Macroscopic lung metastases model. MCA26 cells (3×10^4) were injected in the tail vein 2 days prior to liver tumor implantation. On the day of gene treatment, mice were divided into appropriate control and treatment groups. Some animals were sacrificed for biopsy and pathological examination. One hundred to two hundred tumor nodules were counted on the lung surfaces, with sizes ranging from 0.5 to 0.8 mm in diameter. Histology reports also found tumors in the intestine and mesenteric lymph nodes.

Isolation of mononuclear cells (MNCs) from liver and spleen. The liver or spleen was homogenized and pressed between two semifrosted microscope slides. Dissociated cells were passed through with cell strainer 70 μm (Becton-Dickinson Labware, Flanklin Lakes, NJ) and washed in HBSS containing 1% FBS and the splenocytes were treated with RBC lysis buffer (Sigma, St. Louis, MO). After another wash, the cell pellet was resuspended in 5 ml of wash medium, underlayed with 5 ml of lympholyte-M solution (Cedarlane, Hornby, Canada), and centrifuged at 1000g for 20 min at room temperature. MNCs collected in the interface between lympholyte-M and wash medium were isolated and washed before further analysis.

In vitro cytotoxic assay, depletion, and blocking. The CTL assay required a 5-day stimulation of splenic cells with irradiated parental tumor cells and recombinant mIL-2. The NK assay was performed with freshly isolated MNCs from the liver. The effectors were incubated with ^{51}Cr-labeled target for 4 h at 37°C at various effector-to-target cell ratios. The radioactivity released in the supernatant was measured by a gamma counter and was calculated as follow: (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100. We used Thy-1.2 hybridoma supernatant (ATCC, Rockville, MD) and purified DX5 (Pharmingen, San Diego, CA) or anti-asialo GM1 antibody (Wako Pure Chemical Industries, Ltd., Richmond, VA) with complement to deplete T and NK cells, respectively, under established conditions (11). *In vitro* blocking of the CD3+ effector population was accomplished by purified 145-2C11 antibodies (Pharmingen, San Diego, CA) (11). The standard deviation of triplicate wells was usually less than 7%.

Morphological and immunohistopathological analysis. Mice from tumor-bearing groups before and after various treatments were killed at day 7 following virus injection, and their livers were collected for analysis. Autopsies on long-term tumor-free animals (120 days) were performed to find any viable tumor cells. One-half of the tissue (cuts were made through the midsection of tumors) were fixed in 10% buffered formalin and stained with hematoxylin and eosin, and the other half was fixed in tissue -Tek OCT compound (Miles, Elkhart, IN) and stained for immune cell infiltration. Specific cell type CD8+ (53-5.8, PharMingen) and CD4+ (GK 1.5, Pharmingen) were identified by FITC-conjugated monoclonal antibodies and anti-FITC secondary antibody conjugated with alkaline phosphatase. The staining procedure was performed according to standard protocol.

Tumor rechallenge and in vivo lymphocyte depletion. MCA26 cells (7×10^4) were implanted sc in the flanks of long-term surviving mice (>120 days after treatment). Mice were depleted of lymphocytes subsets using purified ascites GK1.5 (ATCC) for CD4+, 2.43 (ATCC) for CD8+, polyclonal antibodies, anti-asialo GM1 for NK cells, and appropriate Ig controls. Mice were given 200 μg of antibody ip per day, beginning 1 day prior to tumor rechallenge. Antibodies for control and NK cell depletion were administered for 5 consecutive days, then every 5 days afterward (days −1, 0, 1, 2, 3, 8, and 13), while antibodies for CD4+ and CD8+ T-cell depletion were given every other day three times and then every 5 days afterward (days −1, 1, 3, 8, and 13) according to established optimal conditions (6, 11). Depletion efficiencies were confirmed by flow cytometry, and depleted subsets of lymphocytes were routinely obtained (>99%).

RESULTS

Cure of Mice with Advanced Liver Metastases of Colon Cancer by Synergistic Effects of IL-12 Gene Therapy and 4-1BB Costimulation

With the liver being the major organ of metastases for most human gastrointestinal tumors, we have generated a hepatic colon metastases model by direct intrahepatic implantation of a low immunogenic mouse syngeneic colon carcinoma, MCA 26. Seven days after injection, liver tumors measuring 5×5 mm^2 in diameter were directly injected with Adv.mIL-12 or control vector DL312. To improve the long-term antitumor effect of Adv.mIL-12 gene therapy, we combined it with ip administration of an agonistic anti-4-1BB antibody. After 120 days, 80–100% of mice in the combination treatment group (at Adv.mIL-12 viral dose ranging from 0.2×10^8

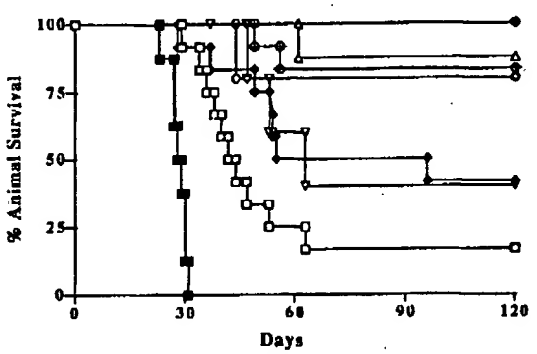

FIG. 1. Survival of tumor-bearing animals after Adv.mIL-12 + anti-4-1BB treatment. MCA26 cells (7×10^4) were implanted into the liver of syngeneic BALB/c mice. After 7 days, mice with hepatic tumor measuring 5×5 mm^2 in diameter were randomly assigned to the following groups: ●, Adv.mIL-12 (3.2×10^8 pfu) + anti-4-1BB ($n = 8$); △, Adv.mIL-12 (1.6×10^8 pfu) + anti-4-1BB ($n = 8$); ⊕, Adv.mIL-12 (0.8×10^8 pfu) + anti-4-1BB ($n = 12$); ◇, Adv.mIL-12 (0.4×10^8 pfu) + anti-4-1BB ($n = 13$); ○, Adv.mIL-12 (0.2×10^8 pfu) + anti-4-1BB ($n = 13$); ▽, Adv.mIL-12 (0.1×10^8 pfu) + anti-4-1BB ($n = 5$); ◆, Adv.mIL-12 (3.6×10^8 pfu) + rat Ig ($n = 12$); □, DL312 (3.6×10^8 pfu) + anti-4-1BB ($n = 12$); and ■, DL312 (3.6×10^8 pfu) + rat Ig ($n = 6$). Antibodies (50 μg/mouse) were given ip at days 8 and 10. The survival advantage for the IL-12 + anti-4-1BB-treated animals, even with an 18-fold lower dose of Adv.mIL-12 (0.2×10^8 pfu), was statistically significant compared to DL312 + anti-4-1BB- or Adv.mIL-12 + control Ig-treated animals ($P = 0.03$, log-rank test). The reported results were combined from three consecutive sets of experiments.

to 3.2×10^8 pfu) were alive and disease-free. In the control group, however, only 35% of the Adv.mIL-12 (3.6×10^8 pfu) plus control Ig- and 14.5% of the DL312 (control vector) plus anti-4-1BB-treated animals were alive (Fig. 1). The results show that the combination therapy requires much less Adv.mIL-12 virus and is synergistically more effective than either treatment alone ($P < 0.0001$, log-rank test).

Systemic Antitumor Immunity Generated by Hepatic IL-12 Gene Treatment Combined with Anti-4-1BB

To further evaluate the systemic antitumor immunity generated by the combination therapy, animals with multiple macroscopic lung metastases (100–200 tumor nodules, 0.5–0.8 mm in diameter) and hepatic tumor were subjected to the test. Animals were treated with a low dose of Adv.mIL-12 (0.4×10^8 pfu) delivered in the liver tumor and then with ip administration of anti-4-1BB. All control-treated animals developed large tumors in the liver, lung, and lymph nodes and died within 32 days, while animals receiving the combination treatment continued to live well after 120 days (Fig. 2). Pathological pictures of liver and lung tumors before treatment are shown in Figs. 3A and 3B, respectively. There was no tumor in the lung, liver, and whole body by histological examination of long-term surviving animals after combination treatment (Figs. 3C and 3D). These results strongly suggest that the systemic antitumor immunity generated from the hepatic IL-12 gene therapy combined with systemic delivery of anti-4-1BB is capable of eradicating preexisting distant lung metastases ($P = 0.0011$, log-rank test).

Effects of IL-12 and Anti-4-1BB on NK Cell Activation

To define the mechanisms underlying the synergistic action between IL-12 and anti-4-1BB, we performed a kinetic analysis of cytolytic activity of MNCs in the liver 0, 2, 4, 7, and 14 days after treatment. The combination-treated animals exhibited a much stronger cytolytic response than the Adv.mIL-12- or anti-4-1BB-only-treated animals (Fig. 4A). It has been reported NK (NK 1.1$^+$) and NKT (NK1.1$^+$/Thy1$^+$) cells are the two major effector cells for the IL-12-activated innate immune response (24). It would be of interest to know what kinds of effector cells are involved in the combination treatment. To identify the responsible cell types, we performed *in vitro* depletion of various leukocyte subsets of MNCs isolated from the animals 2 days after treatment delivery. Depletion of NK cells (Dx5) completely abolished the observed cytolytic activity, while depletion of total T (Thy-1.2) cells only reduced this activity by half, and depletion of CD4$^+$ T (GK 1.5) cells did not affect the killing (Fig. 4B). NK depletion almost completely eliminates the cytolytic effect and T-cell depletion with the pan T-cell marker Thy-1.2 can

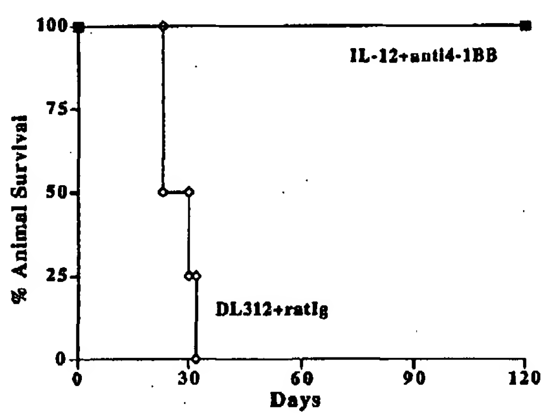

FIG. 2. Effect of combination treatment Adv.mIL-12 of the hepatic tumor + systemic delivery of anti-4-1BB on macroscopic lung metastases of colon carcinoma. *Animals with both liver and lung metastases were used to test the* systemic antitumor effect of the combination IL-12 + anti-4-1BB. Only the liver tumor received IL-12 gene treatment. One hundred percent of the animals treated with DL312 control vector + control Ig ($n = 4$) developed multiple metastases in the lungs, gastrointestinal tract, and liver and died within 32 days. One hundred percent of the animals receiving the Adv.mIL-12 (0.4×10^8 pfu) + anti-4-1BB ($n = 6$) survived well after 120 days. These results indicate a distant protection against preexisting macroscopic lung metastases by hepatic tumor combination treatment ($P = 0.0011$, log-rank test).

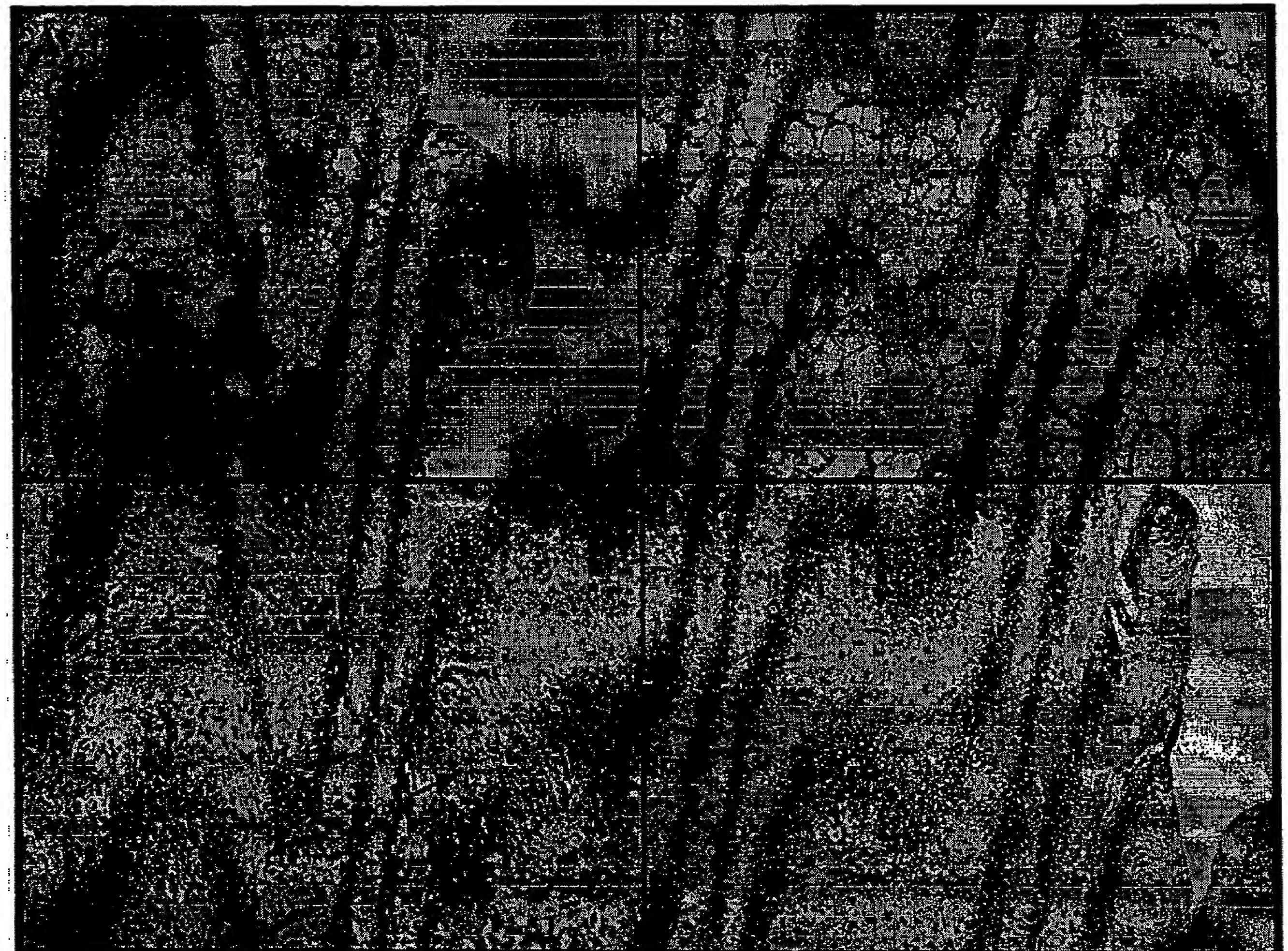

FIG. 3. Histopathological analysis of lung and hepatic metastases of colon carcinoma after combination IL-12 gene therapy + anti-4-1BB. Formalin-fixed and paraffin-embedded sections were stained with hematoxylin and eosin. Magnifications (×100 and ×400) of representative sections of lung metastases (A) and hepatic tumor (B) before treatment are shown. Multiple tumor nodules with a high mitotic index are present in the lung and liver as indicated by arrows. No viable tumor is present in the lung (C) and liver (D) 120 days after combination treatment. Only fibrotic tissue at the tumor injection site was visible in the liver of treated animals (D).

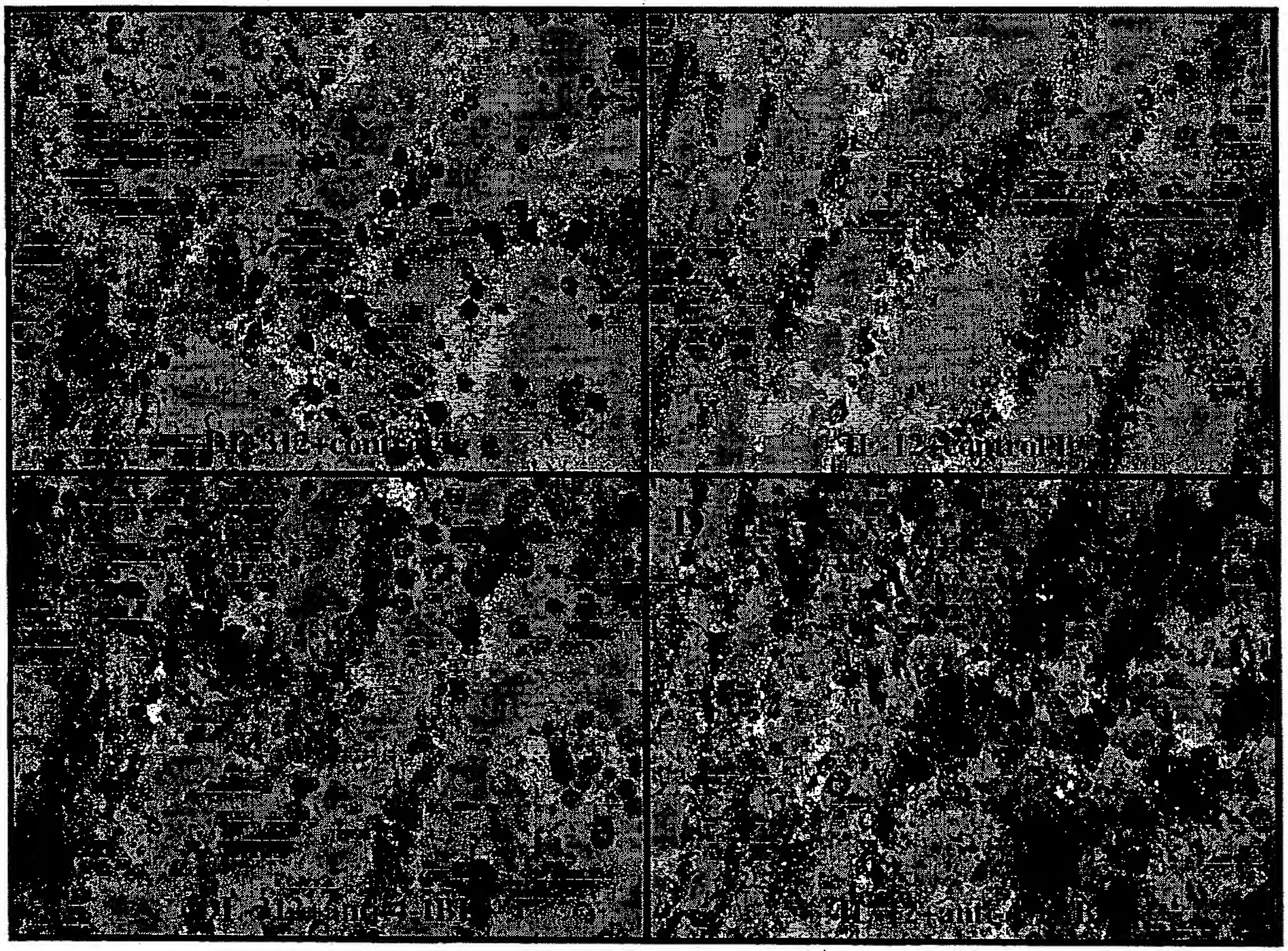

FIG. 6. Immunostaining of CD8+ T cells in MCA26 liver tumor after various treatments (H & E counterstaining, ×400). Distribution of CD8+ T cells at the periphery of MCA26 liver tumor injected with (A) DL312 (no CD8+-positive cells); (B and C) Adv.mIL-12 alone and anti-4-1BB alone, respectively (scattered CD8+-positive cells); and (D) IL-12 + anti-4-1BB (sheets of CD8+-positive cells).

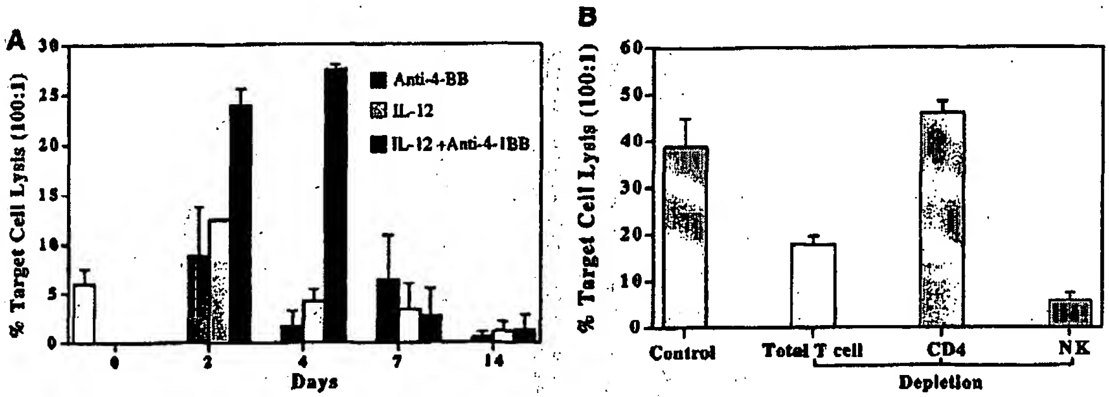

FIG. 4. (A) Evaluation of the NK cell immune response in Adv.mIL-12 (0.4×10^8 pfu) + anti-4-1BB-treated animals. MNCs were isolated from animals at days 0, 2, 4, 7, and 14 (5 mice/group/time point) after treatment and assayed for direct cytolysis against ^{51}Cr-labeled parental MCA26 tumor target. Direct killing activities were seen at days 2 and 4 in ADV.mIL-12 + anti-4-1BB-treated animals (black), but not in animals treated with anti-4-1BB (gray) or Adv.mIL-12 alone (white). The empty column represents the results obtained with MNCs from untreated tumor-bearing animals. (B) Identification of the immune effectors by *in vitro* cell depletion. MNCs isolated from combination-treated animals at day 2 were depleted by complement lysis of NK (DX5), CD4+ T (GK1.5), or pan-T (Thy-1.2) cells. The controls were treated with complement alone. NK cell depletion abolished most of the lytic activity, while total T-cell depletion reduced only by half, and CD4+ T-cell depletion remained constant.

eliminate half of the cytolytic activity. The results indicate that NK cells, and perhaps also some NKT cells with both NK- and T-cell markers, are involved in the antitumor immune response and are synergistically enhanced by the combination treatment at an early time point.

Effects of the IL-12 and Anti-4-1BB Combination Treatment on CTL Development

We also followed CTL development after combination therapy, as it is essential to the persistent immunity and long-term survival of treated animals. A CTL assay against parental MCA26 targets was performed using splenic effector cells isolated from various treatment groups at days 7, 14, 24, and 60 after gene therapy. There was no significant CTL activity in the IL-12-treated animals as reported previously (11). Anti-4-1BB-treated animals exhibited a quite strong CTL response at the early time points. This CTL response vanished with time, however, and led to death of the animals between days 40 and 60 as a result of tumor progression. The cytolytic activities of the combination-treated animals were lower than that of anti-4-1BB alone in the beginning, but gradually increased with time. By day 60 after viral treatment, only the combination treatment animals remained alive. All animals from this group exhibited a strong antitumor CTL activity, which could be completely blocked by CD3 specific antibody, indicating that T cells mediate the killing (Fig. 5). This CTL activity was also tumor-specific because it could not induce lysis of syngeneic heterologous control tumor cells (data not shown). The results strongly suggest that agonistic anti-4-1BB antibody elevates and maintains the T-cell-mediated antitumor immunity induced by Adv.mIL-12 gene therapy.

This synergistic activation of leukocyte subsets was also observed on *in situ* immunostaining of CD8+ in liver tumors of combination-treated animals (Fig. 6). Animals from various treatment groups were sacrificed at day 7 after treatment. No T-cell infiltration and few CD8+ cells were observed in the tumors of DL312 + control Ig- (control group) and in the Adv.mIL-12 + control Ig-treated animals, respectively. More CD8+ T cells were present in DL312 + anti-4-1BB-treated tumors. Interestingly, there was a more robust CD8+ T-cell infiltration in the tumors of Adv.mIL-12 + anti-4-1BB-treated animals than in either treatment alone (Fig. 6). However, there was no significant difference in CD4+ T-cell infiltration among the animals of different treatment groups (data not shown).

CD8+ and NK Cells Are Required for the Persistence of Long-Term Antitumor Immunity

To confirm the cell types responsible for maintenance of antitumor activity and long-term survival, we performed *in vivo* leukocyte subset depletion prior to sc challenge with parental MCA26 tumor cells in long-term surviving animals after combination treatment. Long-term surviving animals injected with control Ig prior to sc tumor challenge served as controls. Tumor growth was observed over a 4-week period. One hundred percent (8/8) of naive mice and 14.2% (1/7) of long-term surviving mice treated with control Ig formed sc tumors. In the NK- and CD8+-cell-depleted groups, 87.5% (7/8) and 100% (8/8) of the animals formed sc tumors, respectively. These results indicate that both NK ($P = 0.0106$) and CD8+ T cells ($P = 0.0005$, Fisher's exact test) are essential in protecting surviving animals from tumor relapse after Adv.mIL-12 + anti-4-1BB therapy.

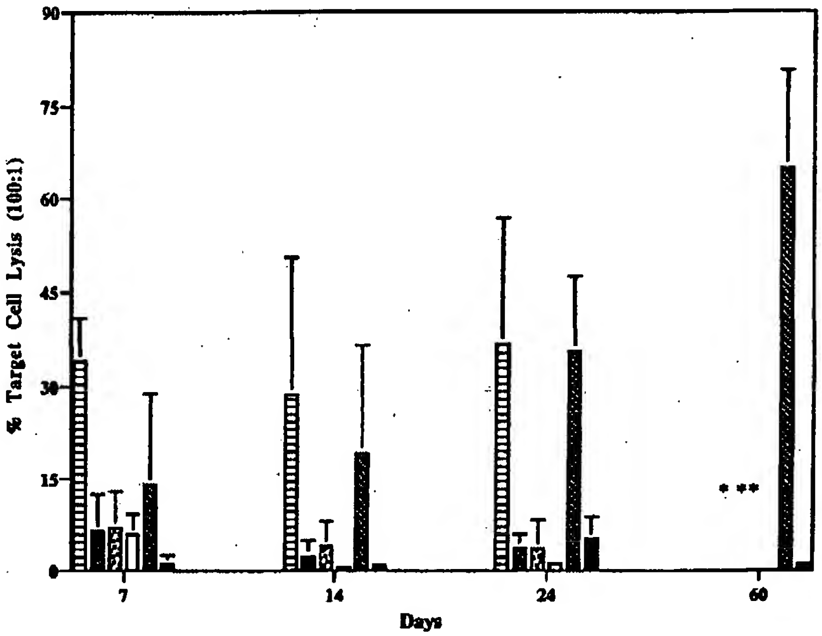

FIG. 5. Evaluation of the CTL immune response in Adv.mIL-12 (0.4×10^8 pfu) + anti-4-1BB-treated animals. CTL analysis was performed on individual animals ($n = 5$–6/group/time point) at days 7, 14, 24, and 60 after treatment. Significant cytolytic activity against ^{51}Cr-labeled parental MCA26 target was observed in the anti-4-1BB antibody alone- (horizontal line) or combination IL-12 + anti-4-1BB-treated animals (right hatched) but not in Adv.mIL-12 alone-treated animals (dotted). There was a gradual and significant increase of CTL activity in the combination-treated animals over time. *In vitro* blocking of $CD3^+$ effector completely abolished the cytolytic response induced by the combination treatment (filled) or anti-4-1BB alone (empty), but had no effect on the response induced by IL-12 alone (left hatched). By day 60, all animals treated with anti-4-1BB (*) or Adv.mIL-12 (**) alone were dead, while those in the combination treatment group remained healthy and exhibited persistent CTL.

DISCUSSION

Using an Adv.mIL-12 and anti-4-1BB agonistic antibody combination therapy, we achieved eradication and long-term remission of both hepatic and multiple macroscopic lung metastases in a mouse model of metastatic colon carcinoma. This is the first report to show a synergistic effect of 4-1BB and IL-12 with potentiation of both early NK antitumor response and long-term CTL. Importantly, this combination therapy allowed an 18-fold reduction of the IL-12 dose compared to the effective dose of IL-12 alone and achieved better efficacy than either treatment alone. This lower dose of IL-12 provides a safety advantage for IL-12-mediated gene therapy. IL-12 in combination with other cytokines or costimulatory molecules has been extensively studied in various animal models of cancer (22–25). However, similar significant results as those shown here with the combination Adv.mIL-12 plus anti-4-1BB antibody have never been reported.

The treatment-induced long-term antitumor immunity requires both NK and $CD8^+$ T cells, as shown in the challenge experiment of surviving animals after combination treatment. Furthermore, some direct NK activity (10–15% killing) was detected in the NK assay *in vitro* with hepatic MNCs from long-term surviving animals after combination treatment.

The 4-1BB signal has been shown to induce proliferation of activated T cells and to amplify preferentially the cytotoxic T-cell response (19). However, in our orthotopic metastatic model the CTL response induced by anti-4-1BB alone did not persist, resulting in death of most of the animals due to tumor relapse or tumor progression. The treatment of tumor-bearing animals with different doses (200, 100, and 50 μg) of anti-4-1BB did not statistically improve their survival rate (data not shown), demonstrating that activated T cells cannot be further expanded by anti-4-1BB alone. Thus, initiation of T-cell activation and T-cell priming appear to be the limiting steps for the development of persistent CTL.

Whether 4-1BB directly or indirectly works on IL-12-activated NK or NKT cells has yet to be elucidated. The bridge between IL-12-activated innate immunity and adaptive immunity is important. There is evidence that demonstrates the critical link between NK and CTL development. NK cells have been shown to play a crucial role in the generation of antitumor T cells (26, 27) and

the NK-cell-secreted cytokines (IL-2 and IFN-γ) may be involved in CTL development. The persistent antitumor specific CTL activity observed only in the combination Adv.mIL-12 + anti-4-1BB-treated animals may be explained by 4-1BB engagement on IL-12-activated NK cells, leading to NK secretion of appropriate cytokines and promotion of APC activation (28). APCs work directly on or assist helper T-cell development in promoting CTL proliferation and persistence. Although anti-4-1BB antibodies per se can induce a significant short-term CTL activity, just 14.5% of the anti-4-1BB-only-treated animals show long-term survival; anti-4-1BB antibody-activated T cells may not persist because of a less efficacious antigen presentation and priming.

Other mechanisms involved in the IL-12-induced antitumor response include antiangiogenesis mediated by IFN-γ and IP-10 (29), upregulation of Fas-mediated tumor apoptosis (30), and cytokine-mediated cytotoxicity effect to suppress tumor proliferation (31). However, all of these IL-12-mediated antitumor effects probably play only an early role and will vanish with time. The tumor will recur or continue to progress because of the transient expression of Adv.mIL-12. The results of our study indicate that the systemic and persistent antitumor immunity induced by the Adv.mIL-12 + anti-4-1BB treatment is the major mechanism for long-term survival of tumor-bearing animals. Interestingly, our subcutaneous challenge experiment with parental tumor cells has shown that the persistent antitumor immunity involves both CTL and NK cells. Whether this persistent NK response is NK- or NKT-mediated requires further clarification.

Further studies of the mechanism of cellular and cytokine regulation mediated by the combination treatment will improve our scientific understanding of immune-modulatory gene therapy for clinical application in metastatic carcinomas in humans. The combination of IL-12 and anti-4-1BB antibody may provide a new treatment modality for patients with metastatic colon cancer.

ACKNOWLEDGMENTS

We thank Drs. Simon Hall and Bernhard Sauter for their helpful discussion on this project. The work was partially supported by grants from the National Cancer Institute (R29 CA-70337, R01 CA-75175, and CA-84404 to S.H.C.; RO1 CA 79915 to L.C.) and Novartis Stiftung (to O.M.).

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